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Differential Effects of the Oncogenic BRAF Inhibitor PLX4032 (Vemurafenib) and its Progenitor PLX4720 on ABCB1 Function

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ABSTRACT PURPOSE: The clinically approved oncogenic BRAF inhibitor PLX4032 (vemurafenib) was shown to be a substrate of the ATP-binding cassette (ABC) transporter ABCB1. Here, we compared PLX4032 and its structurally closely related precursor compound PLX4720 for their interference with ABCB1 and the ABCB1-mediated compound transport using docking and cell culture experiments. **METHODS:** For the docking study of PLX4032 and PLX4720 with ABCB1, we analysed binding of both compounds to mouse Abcb1a and to human ABCB1 using a homology model of human ABCB1 based on the 3D structure of Abcb1a. Naturally ABCB1 expressing cells including V600E BRAF-mutated and BRAF wild-type melanoma cells and cells transduced with a lentiviral vector encoding for ABCB1 were used as cell culture models. ABCB1 expression and function were studied by the use of fluorescent and cytotoxic ABCB1 substrates in combination with ABCB1 inhibitors. **RESULTS:** Docking experiments predicted PLX4032 to interact stronger with ABCB1 than PLX4720. Experimental studies using different cellular models and structurally different ABCB1 substrates confirmed that PLX4032 interfered stronger with ABCB1 function than PLX4720. For example, PLX4032 (20 µM) induced a 4-fold enhanced rhodamine 123 accumulation compared to PLX4720 (20 µM) in ABCB1-transduced UKF-NB-3 cells and reduced the IC₅₀ for the cytotoxic ABCB1 substrate vincristine in this model by 21-fold in contrast to a 9-fold decrease induced by PLX4720. **CONCLUSIONS:** PLX4032 exerted stronger effects on ABCB1-mediated drug transport than PLX4720. This indicates that small changes in a molecule can substantially modify its interaction with ABCB1, a promiscuous transporter that transports structurally different compounds.

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INTRODUCTION

Metastatic melanoma belongs to the most aggressive cancer diseases with median survival times below 9 months. Activating BRAF (V600E) mutations are found in 30 to 70% of melanoma patients representing a novel therapeutic target for this disease (1). PLX4032 (vemurafenib), a specific inhibitor of V600E-mutated BRAF, caused high response rates and advanced overall and progression-free survival rates in metastatic melanoma patients carrying V600E BRAF mutations. Unfortunately, resistance to PLX4032 develops quickly (2).

PLX4032 was also found to be a substrate of the ATP-binding cassette (ABC) transporter ABCB1 (also known as P-glycoprotein or MDR1) (3-5). The interaction of drugs with ABCB1 is important because this transporter plays important roles in the passage of drugs, xenobiotics, and food

constituents through cellular and tissue barriers and consequently in their absorption, distribution, and excretion. The clinical investigation of ABCB1 inhibitors had revealed that the use of compounds that interfere with ABCB1 function may result in toxic adverse events and complex pharmacokinetics and drug interactions that are difficult to predict (6,7). Moreover, ABCB1 is one of the most intensively studied intrinsic and acquired resistance mechanisms in cancer cells. It is known to cause resistance to major classes of anti-cancer drugs including vinca alkaloids, taxanes, and anthracyclines (6,7).

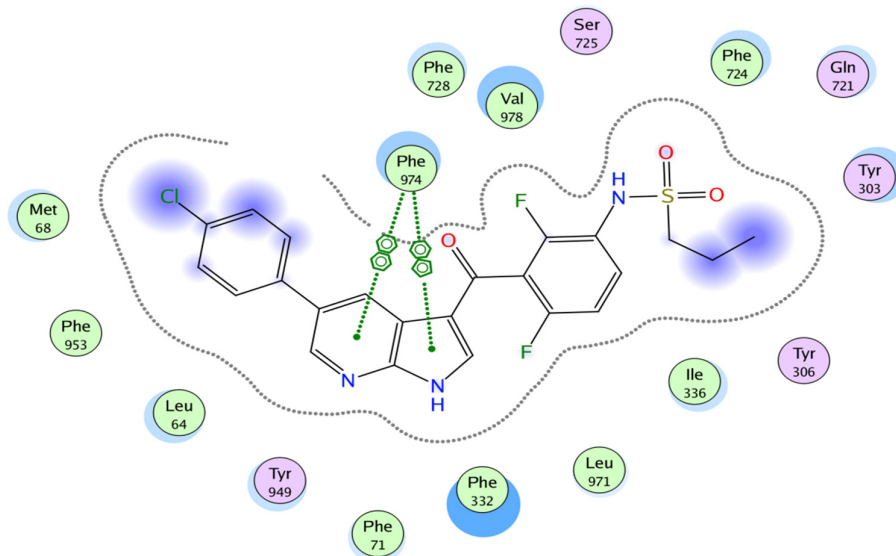
PLX4720 is a structurally related progenitor of PLX4032 that displays the same mode of binding to V600E-mutated BRAF like PLX4032 (8,9).

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It differs only in one phenyl ring from PLX4032 (Figure 1). Here, we performed the first study that compared the effects of these two compounds on ABCB1 function. Docking studies suggested both PLX4032 and PLX4720 to bind to ABCB1 with

PLX4032 displaying a slightly higher affinity. Verification experiments performed using a range of different cellular models and assays confirmed that PLX4032 exerts stronger effects on ABCB1 than PLX4720.

A



B

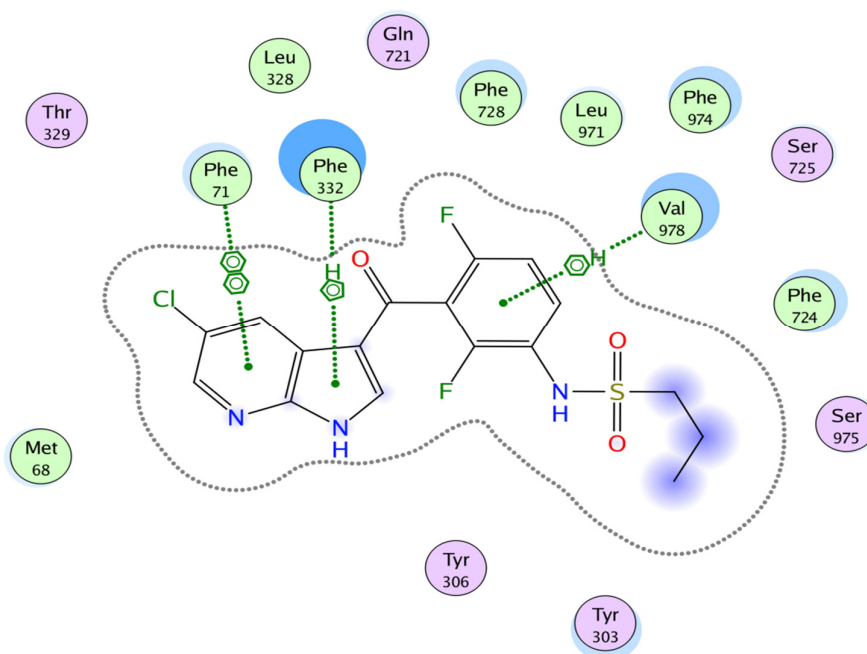


Figure 1. 2D ligand interaction diagram for PLX4032 (A) and PLX4720 (B) with mouse Abcb1a using MOE software. In this diagram, the energy cut-off for H-bond and ionic interactions were -0.5 kcal/mol and the maximum distance for nonbonded groups was 4.5 Å. The polar and non-polar interactions are indicated by pink or green coloured amino acids and hydrogen bonding is indicated by green dotted arrows. The proximity contour is the dotted line surrounding the ligand. It indicates the shape of the binding site and the available space to the more outward-facing parts of the ligand. Blue shadows in some amino acids indicate the receptor exposure differences by the size and intensity of the quito discs. The directions of the shadow indicate the directions of the amino acids towards the ligands. The blue clouds around the ligand atoms indicate the solvent exposure.

MATERIALS AND METHODS

Drugs

Vincristine, verapamil, and PGP-4008 were obtained from Sigma-Aldrich (Deisenhofen, Germany). Rhodamine 123 was purchased from Merck Biosciences (Darmstadt, Germany). PLX4032 and PLX4720 were obtained from Selleck Chemicals via BIOZOL GmbH (Eching, Germany). Cisplatin was purchased from medac Gesellschaft für klinische Spezialpräparate mbH Wedel, Germany).

ABCB1 docking studies

The structure of Abcb1a, the mouse (*Mus musculus*) ortholog of the human ABCB1, had been co-crystallised with two stereo-isomers of cyclic hexapeptide inhibitors, cyclic-tris-(R)-valineselenazole (QZ59-RRR) and cyclic-tris-(S)-valineselenazole (QZ59-SSS) (10,11). For the docking study of PLX4032 and PLX4720 with ABCB1, we applied different approaches. We analysed binding of both compounds to mouse Abcb1a and (since high or medium resolution X-ray structures of human ABCB1 are not available) to a homology model of human ABCB1 based on the 3D structure of Abcb1a (12). The X-ray structure of the mouse Abcb1a was downloaded from the protein data bank [<http://www.rcsb.org>]. Docking was carried out using 1) the MOE software (MOE, version 2010.10, Chemical Computing Group Inc. Montreal, Canada) and 2) the web application at <http://pgp.althotas.com> (12) which performs docking using the Autodock Vina software integrated in the Molecular Docking Server (<http://www.dockingserver.com>).

The Mouse Abcb1a structure was loaded into the MOE software, where the crystal parameters were retained and hydrogens were added. Protonatable residues were titrated using default parameters. To prepare PLX4032 and PLX4720 for docking, atomic charge and energy minimisation calculation was performed using SCF optimization through AM1 semiempirical method as implemented in the MOE software. In the MOE dock panel, the placement method was Triangle Matcher. The scoring methodology was set to London dG as the first and the second scoring functions. The refinement methodology was set to Forcefield, and finally, the 30 best scoring poses and the mean energies were retained. The binding site was defined in MOE software using the co-crystallised ligand QZ59-RRR. The published X-ray crystallography structures (10,11) were used to validate our docking model by comparing the

geometries of the docked Abcb1a/QZ59-RRR structure and the structure of the Abcb1a/QZ59-RRR complex from X-ray crystallography. The root-mean-square deviation (RMSD) value for this structure after superposing the docked and co-crystal structure is 0.77. The absolute RMSD range without superposing is 0.89-6.2 for the top 30 poses. Moreover, PDB structures of the ligands were prepared using MOE software and fed into the web server at <http://pgp.althotas.com>.

Cell lines

The neuroblastoma cell line UKF-NB-3 was established from bone marrow metastases of a neuroblastoma patient with MYCN-amplification (13). Its sub-line UKF-NB-3^{VCR}¹⁰ was established by its adaptation to growth in the presence of vincristine (10ng/mL) and is characterised by high ABCB1 expression (13,14). ABCB1-overexpressing (UKF-NB-3^{ABCB1}, control cell line UKF-NB-3^{control}) sub-lines of UKF-NB-3 were established by lentiviral transduction with LeGO vectors as described previously (14-17). Vectors were derived using standard molecular cloning techniques as described previously (13,14, see also www.lentigo-vectors.de).

The melanoma cell lines Colo-679, IPC-298, MelHO, RVH-421, and SK-Mel-30 were obtained from DSMZ (Braunschweig, Germany).

All cell lines were propagated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 100IU/ml penicillin and 100mg/ml streptomycin at 37°C.

Viability assay

Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay after 120h incubation, modified as described previously (14,17). All experiments were performed at least in triplicate.

Flow cytometry and investigation of ABC transporter-mediated drug efflux

An antibody directed against ABCB1 (Alexis Biochemicals via AXXORA Deutschland, Lörrach, Germany) and a secondary antibody labelled with Phycoerythrin (R&D, Wiesbaden, Germany) were used to detect ABCB1 expression by flow cytometry (FACSCalibur, BD Biosciences, Heidelberg, Germany).

The ABCB1-mediated drug efflux and wash-out kinetics were determined as described before (14,17). To investigate ABCB1-mediated substance efflux, the cells were pre-incubated for

30min with different drug concentrations. PGP4008 was used as control inhibitor of ABCB1-mediated efflux. 0.1 μ M rhodamine 123 was added for another 30 min. Then, cell culture medium was removed, cells were washed three times with PBS, and fresh medium containing drugs was added. After another 45min, the cellular fluorescence was analysed by flow cytometry. Rhodamine 123 was detected at the FL1 channel.

For wash out experiments, UKF-NB-3^{ABCB1} cells were incubated for 1h with rhodamine 123 0.1 μ M together with different drug concentrations or with PGP4008. Subsequently, cells were washed, resuspended in supplemented medium, and the cellular fluorescence was measured at different time points (t_0 , t_5 , t_{15} , t_{30} , t_{60} , t_{120} minutes) by flow cytometry.

Determination of ATPase activity

The ATPase activity of ABCB1 was determined using membrane preparations (BD Biosciences, Heidelberg, Germany) and an established kit (BD Biosciences, Heidelberg, Germany) following the manufacturer's instruction.

STATISTICAL ANALYSIS

Two groups were compared by t-test. More groups were compared by ANOVA with subsequent Student-Newman-Keuls test.

RESULTS

Docking studies on the interaction of PLX4032 and PLX4720 with ABCB1

Two docking approaches were used (web server docking, MOE) to predict the interaction of PLX4032 and PLX4720 with ABCB1. These approaches were applied using the X-ray structure of mouse Abcb1a, the mouse ortholog of the human ABCB1, or a homology model of human ABCB1.

The interactions of PLX4032 and PLX4720 with mouse Abcb1a are illustrated in Figure 1. Most amino acids in the active site are lipophilic. For PLX4032, docking energies in the top ten poses are quite high; energy of the top pose is -14.6 according to Table 1. Ligand interactions of the top ten poses showed that Phe974 is actively involved in several poses. Further examination of the top ten poses renditions indicated that the placement of the π - π interaction of Phe974 is qualitatively preserved. Figure 1A is the structure of the top pose and shows the pyridine and pyrrole rings in the pyrrolo-pyridine in a strong arene-arene interaction to Phe 974. The large and intense blue shadows surrounding the amino acids Phe 332, Val 978, Tyr 303, Gln 721, Phe 724, and Phe 974, indicate that in the absence of the ligand these amino acids are highly exposed to the solvent, but the presence of the ligand greatly reduces the solvent accessible surface area. The amino acids Met 68, Leu 64, and Tyr 949 are also involved but to a lesser extent. The chlorobenzene group on the left as well as the propyl side chain on the sulphonamide illustrate degrees of solvent exposure (Figure 1A). In PLX4720, the propane sulphonamide group is also exposed to solvent (Figure 1B). The difluoroaniline has a strong arene-hydrogen bond interaction to Val 978, while the chloro-pyrrolo pyridine (left) has an arene-arene interaction to Phe 71 through the pyridine ring and arene-hydrogen bond to Phe 332 through the pyrrole ring. The receptor exposure differences are the highest for Phe 332 and Val 978 (Figure 1B).

The calculated docking energies for the 30 top poses are presented in Table 1 for mouse Abcb1a and for human ABCB1. PLX4032 has a stronger interaction energy with both forms, as indicated by comparing the energies of the top pose and the average of the 30 top poses using both docking methods (web server docking, MOE) (Table 1).

Table 1. Protein interaction energies (kcal/mol) of top scoring poses of docking mouse Abcb1a or human ABCB1 with PLX4032 and PLX4720 by two different approaches. The average of the 30 top scoring poses is reported; values in brackets are the minimum and the maximum energies.

Receptor	web server docking (http://pgp.althotas.com/)		MOE	
	PLX4032	PLX4720	PLX4032	PLX4720
mouse Abcb1a	-10.7	-9.0	-10.6 (-14.6, -6.72)	-9.91 (-13.73, -5.86)
human ABCB1	-11.2	-10.3	-9.10 (-11.0, -7.42)	-8.78 (-10.1, -8.26)

The distances between the important groups in PLX4032 or PLX4720 and the interacting residues in mouse Abcb1a are shown in Table 2. Despite that docking scores are often not very well correlated with the actual biological affinity, given that PLX4032 has one additional aromatic ring in comparison with PLX4720 and this can potentially improve binding affinity in the absence of steric hindrance, taken together, the docking results indicate a stronger interaction with ABCB1 for PLX4032 than for PLX4720.

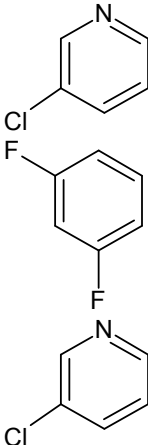
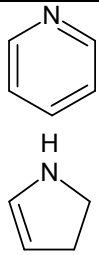
Effects of PLX4032 and PLX4720 on ABCB1 function

PLX4032 and PLX4720 both enhanced accumulation of the fluorescent ABCB1 substrate rhodamine 123 in UKF-NB-3^{ABCB1} cells (but not in empty vector-transduced control UKF-NB-3^{Ctrl} cells or non-transduced parental UKF-NB-3 cells

that lack ABCB1 expression, not shown) in a concentration-dependent manner (Figure 2A). PLX4032 (20 μ M) induced a 4-fold enhanced rhodamine 123 accumulation compared to PLX4720 (20 μ M). Neither PLX4032 nor PLX4720 affected ABCB1 expression in concentrations up to 20 μ M after incubation for up to 5 days (data not shown).

Next, we determined the rhodamine 123 wash-out kinetics in the presence of PLX4032 or PLX4720. Cells were incubated with rhodamine 123 in the presence of PLX4032 or PLX4720 for 60 min. Subsequently, cells were washed and the cellular fluorescence was monitored at different time points after the wash-out by flow cytometry. The rhodamine 123 fluorescence rapidly decreased after wash-out of PLX4032 or PLX4720 (Figure 2B). Moreover, both compounds increased the ABCB1 ATPase activity (Figure 2C).

Table 2. Distances between groups in PLX4032 or PLX4720 and the corresponding protein residues in mouse Abcb1a as indicated by the MOE docking approach

Ligand	Residue	Group in PLX4720	Distance (Å)
PLX4720	Phe332		3.50
	Val978		4.01
	Phe71		3.61
Ligand	Residue	Group in PLX4032	Distance (Å)
PLX4032	Phe974		3.64
	Phe974		3.99

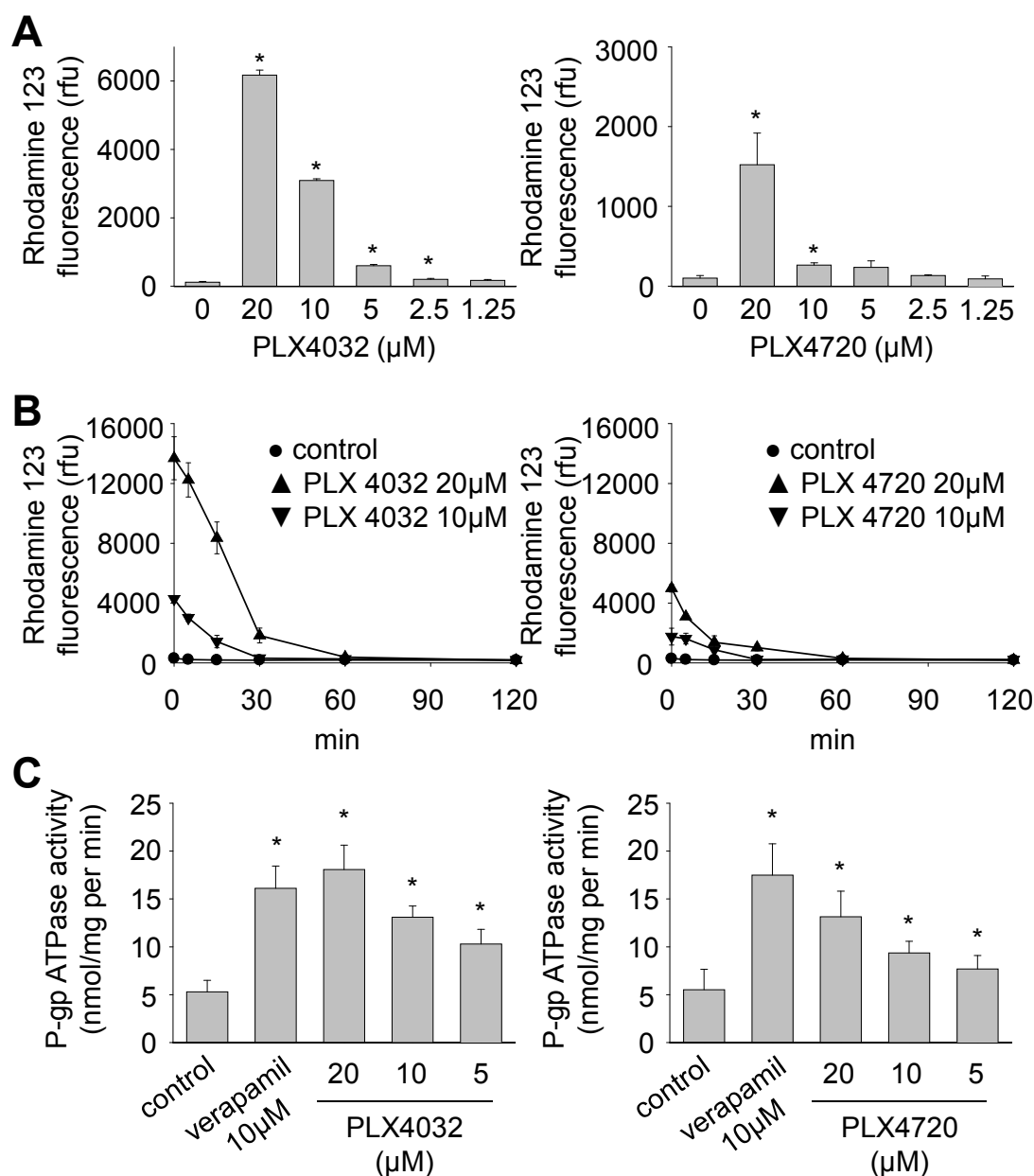


Figure 2. Interference of PLX4032 and PLX4720 with ABCB1 activity. A) Concentration-dependent influence of PLX4032 or PLX4720 on rhodamine 123 (0.1 μM) fluorescence in UKF-NB-3^{ABCB1} cells (Cells were pre-incubated for 30min with the indicated PLX4032 concentrations. Then, 0.1 μM rhodamine 123 was added for another 30 min. After this, cell culture medium was removed, cells were washed three times with PBS, and fresh medium containing PLX4032 was added. After another 45min, the cellular fluorescence was analysed by flow cytometry), B) time kinetics of rhodamine 123 (0.1 μM) fluorescence in UKF-NB-3^{ABCB1} cells in the presence of PLX4032 or PLX4720 after a 60 min pre-incubation period with subsequent wash-out of extracellular rhodamine 123 and PLX4032 or PLX4720 (control = rhodamine 123 incubation in the absence of drugs). C) ABCB1 ATPase activity in isolated membranes in the presence of PLX4032 or PLX4720 (control = activity in the absence of drugs). Verapamil, a known ABCB1 substrate, was used for comparison. * $P < 0.05$ relative to non-treated controls

PLX4032 and PLX4720 also increased the toxicity of the ABCB1 substrate vincristine in UKF-NB-3^{ABCB1} cells in a concentration-

dependent manner (Figure 3, Suppl. Table 1). In accordance with the cellular rhodamine 123 accumulation data, PLX4032 caused substantially

stronger effects than PLX4720 (Figure 3, Suppl. Table 1). Neither PLX4032 nor PLX4720 affected vincristine toxicity in non-transduced UKF-NB-3 cells (Figure 3, PLX4032, Suppl. Table 2; PLX4720, Suppl. Table 3) or in UKF-NB-3 cells transduced with a control vector (Figure 3, PLX4032, Suppl. Table 4; PLX4720, Suppl. Table 5).

Effects of PLX4032 and PLX4720 on melanoma cells in combination with the cytotoxic ABCB1 substrate vincristine

PLX4032 and PLX4720 were further investigated in combination with the cytotoxic ABCB1 substrate vincristine in two melanoma cell lines expressing wild-type BRAF (IPC-298, SK-Mel-30). Both cell lines expressed ABCB1. The ABCB1 inhibitor PGP4008 (Table 3) and PLX4032 and PLX4720 decreased the vincristine IC_{50} s in both cell lines (Table 4). Again, PLX4032 exerted stronger effects than PLX4720.

Effects of PLX4032 in combination with structurally different ABCB1 substrates in different ABCB1 expressing cells

ABCB1 transports a broad spectrum of structurally different substances (6). Therefore, we investigated the effects of PLX4032 and PLX4720 on the sensitivity of the ABCB1-expressing cell lines UKF-NB-3^{ABCB1} and UKF-NB-3^{VCR}¹⁰ to the cytotoxic ABCB1 substrates vincristine, paclitaxel, and actinomycin D. The non-ABCB1 substrate CDDP was used as negative control. Both cell lines were sensitised by both compounds to all three ABCB1 substrates but not to cisplatin (Figure 4, Suppl. Table 6). In UKF-NB-3^{ABCB1} cells, PLX4032 10 μ M reduced the IC_{50} for vincristine by 37.81-fold, for paclitaxel by 7.61-fold, and for actinomycin D by 16.28-fold. PLX4720 10 μ M caused in UKF-NB-3^{ABCB1} cells an IC_{50} reduction by 6.88-fold for vincristine, by 2.73-fold for paclitaxel, and by 3.50-fold for actinomycin D. In UKF-NB-3^{VCR}¹⁰ cells, PLX4032 10 μ M decreased the vincristine IC_{50} by 27.98-fold, the paclitaxel IC_{50} by 4.10-fold, and the actinomycin D IC_{50} by 6.06-fold, while PLX4720 reduced the respective IC_{50} values by 7.19-fold (vincristine), 1.77-fold (paclitaxel), and 3.68-fold (actinomycin D) (Figure 4, Suppl. Table 6). The compounds did neither sensitise UKF-NB-3 cells nor UKF-NB-3 cells transduced with a control vector (serving as vector control for UKF-NB-3^{ABCB1} cells) to toxicity induced by vincristine, paclitaxel,

actinomycin D, or cisplatin (Figure 4, Suppl. Table 7). Taken together, these data show that PLX4032 and PLX4720 interfere with a range of structurally different ABCB1 substrates and that PLX4032 consistently exerts stronger effects on the ABCB1-mediated compound transport.

DISCUSSION

PLX4032, an inhibitor of V600E-mutated BRAF, demonstrated clinical efficacy for the treatment of V600E-mutated melanoma (2) and was shown to be a substrate of ABCB1 (3-5), an ABC transporter that is involved in the regulation of the absorption, distribution, and excretion of drugs and xenobiotics as well as in cancer cell drug resistance (6).

Here, we compared PLX4032 and its structurally related progenitor compound PLX4720 for their effects on ABCB1. First, we performed docking experiments. The interaction energies and the 2D representation of the interaction plot for the top poses indicated that PLX4032 has a stronger interaction with ABCB1 than PLX4720. Both compounds interfere with ABCB1's Phe 332 and Val 978, and in addition PLX4032 has strong interaction with Phe 974.

The docking results were experimentally confirmed using UKF-NB-3 neuroblastoma cells transduced with a lentiviral vector encoding for ABCB1 and ABCB1-expressing UKF-NB-3^{VCR}¹⁰ cells. PLX4032 and PLX4720 interfered with the transport of compounds from structurally different classes including the fluorescence dye rhodamine 123 and the cytotoxic anti-cancer drugs vincristine, paclitaxel, and actinomycin D. These findings are quite remarkable because they show that small changes in a molecule like PLX4032 can result in a substantial change in its affinity to ABCB1 despite the tremendous promiscuity of this transporter that recognises substrates from a broad range of structurally very different compounds (6). Compounds that interfere with ABCB1 function may modify the pharmacokinetics of co-administered substances. This may result in toxic adverse events and unpredictable drug interactions (7). Therefore, compounds that display less interaction with ABCB1 may be favourable, in particular if administered at the same time as cancer-unrelated drugs. The situation may be less clear regarding anti-cancer therapy regimens that contain PLX4032 in combination with other anti-cancer drugs as proposed previously (18).

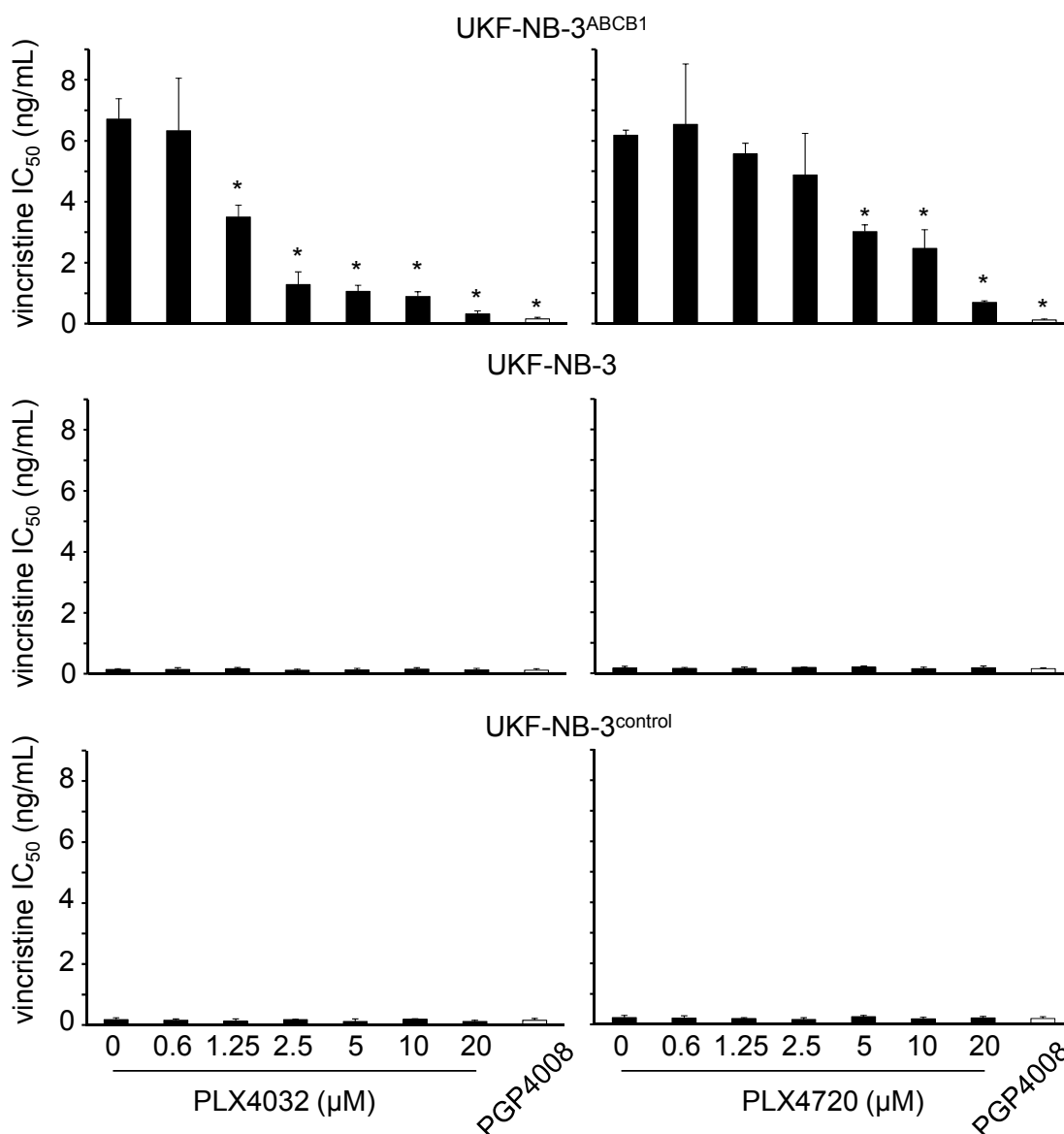


Figure 3. Influence of PLX4032 and PLX4720 on the vincristine sensitivity of UKF-NB-3 cells transduced with a lentiviral vector (UKF-NB-3^{ABCB1}), UKF-NB-3 cells, and UKF-NB-3 cells transduced with an empty lentiviral control vector (UKF-NB-3^{control}). Cell viability was determined after 5 days of incubation by MTT assay. The ABCB1 inhibitor PGP4008 (2.5 μM) served as control. The values are presented in the Suppl. Tables 1-5. * P < 0.05 relative to vincristine alone.

In combination with non-ABCB1 substrates and/or for the treatment of ABCB1-negative cells, the interference of PLX4032 with ABCB1 bears the risk of unfavourable side-effects due to the modification of the body distribution of xenobiotics and cancer-unrelated drugs. However, combinations of PLX4032 with other anti-cancer drugs that are ABCB1 substrates may result in synergistic effects due to inhibition of ABCB1-

mediated drug efflux in ABCB1-expressing cancer cells.

PLX4032 and PLX4720 also enhanced the effects of vincristine in the ABCB1-expressing wild-type BRAF melanoma cell lines IPC-298 and SK-MEL-30. In addition, PLX4032 increased the toxicity of the ABCB1 substrate vincristine in V600E BRAF-mutated melanoma cells (Figure 5) although we did not define the driver mechanisms underlying this combined effect.

Table 4. Influence of PLX4032 (20 μ M) or PLX4720 (20 μ M) on the vincristine (ABCB1 substrate) concentrations that reduce cell viability by 50% (IC₅₀) in the BRAF wild-type melanoma cell lines IPC298 and SK-MEL-30 that both express ABCB1.

	IC ₅₀ vincristine (ng/ml)	IC ₅₀ vincristine in the presence of PLX4032/PLX4720	cell viability in the presence of PLX4032 or PLX4720 alone (% control)	fold sensitisation ¹
PLX4032				
IPC298	2.74 \pm 0.39	0.54 \pm 0.07*	73 \pm 7	5.11
SK-MEL-30	4.57 \pm 1.49	0.42 \pm 0.07*	85 \pm 9	10.82
PLX4720				
IPC298	2.74 \pm 0.39	0.83 \pm 0.08*	57 \pm 10	3.32
SK-MEL-30	4.57 \pm 1.49	1.50 \pm 0.62*	73 \pm 5	3.04

¹ IC₅₀ vincristine/ IC₅₀ vincristine in the presence of PLX4032 or PLX4720, respectively; * p < 0.05 relative to vincristine alone

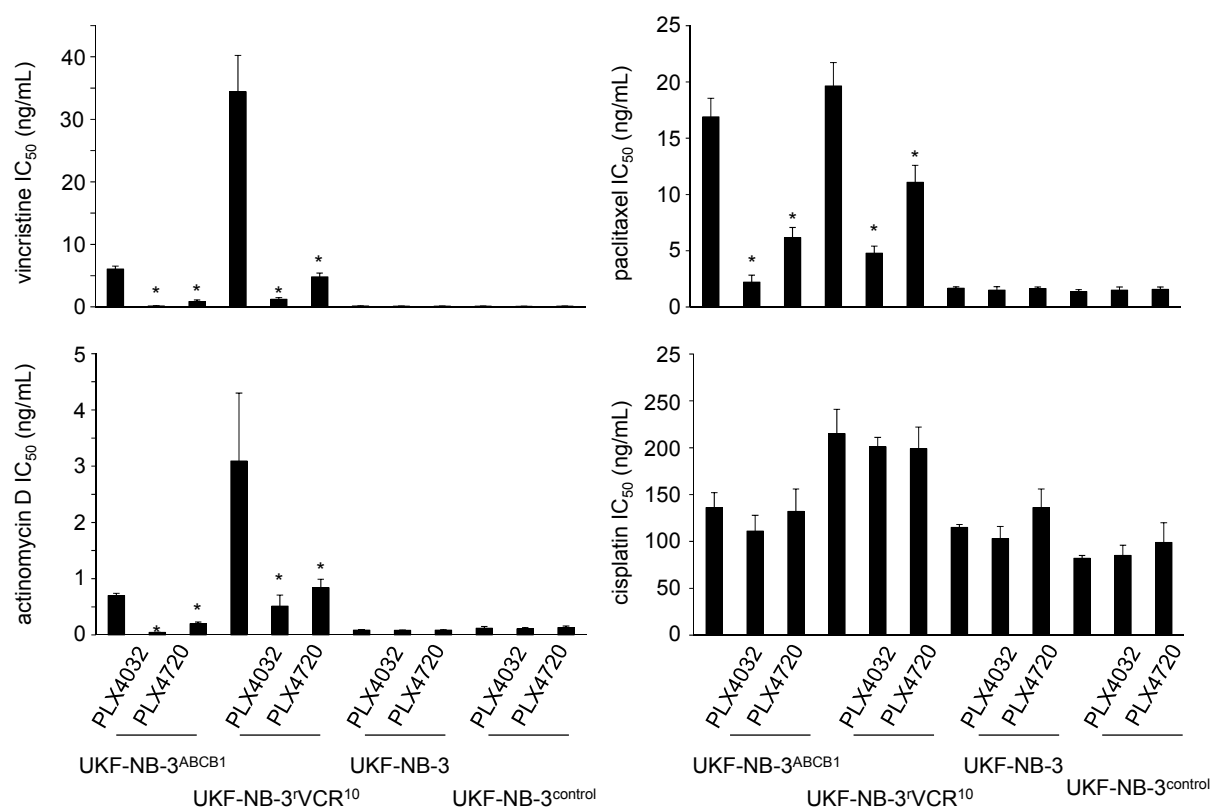


Figure 4. Effects of PLX4032 (10 μ M) and PLX4720 (10 μ M) on the sensitivity of UKF-NB-3 cells transduced with a lentiviral vector (UKF-NB-3^{ABCB1}), ABCB1-expressing UKF-NB-3^{VCR¹⁰} cells, UKF-NB-3 cells, and UKF-NB-3 cells transduced with an empty lentiviral control vector (UKF-NB-3^{control}) to structurally different cytotoxic ABCB1 substrates. Cell viability was determined after 5 days of incubation by MTT assay. Cisplatin that is not an ABCB1 substrate served as control. The values are presented in the Suppl. Tables 6 and 7. * P < 0.05 relative to the respective cytotoxic drug alone.

A rapid decrease of cellular fluorescence mediated by the ABCB1 substrate rhodamine 123

was detected after the wash-out of PLX4032 and PLX4720. Also, both compounds stimulated the

ATPase activities of ABCB1. These findings may suggest that PLX4032 and PLX4720 are ABCB1 substrates. Thus, they are in accordance with recent publications that showed that PLX4032 is a ABCB1 substrate (3,4). Notably, the interaction of compounds like PLX4032 and PLX4720 with ABCB1 may depend on the cellular context since PLX4720 was recently shown not to affect ABCB1 function in Ras-NIH 3T3/Mdr cells (19).

In conclusion, we show that PLX4032 exerts stronger effects on ABCB1 than its structurally very similar progenitor PLX4720. The findings demonstrate that small changes in a molecule can substantially modify its interaction with ABCB1 although ABCB1 is an extremely promiscuous transporter that transports a broad range of structurally different compounds.

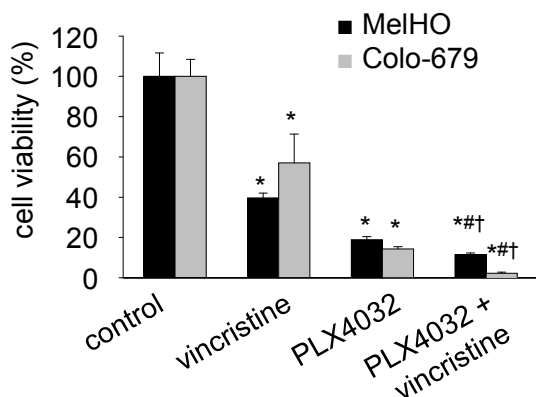


Figure 5. Influence of PLX4032 on the sensitivity of ABCB1- and ABCC1-expressing V600E BRAF-mutated melanoma cells to the cytotoxic ABCB1 and ABCC1 substrate vincristine. Viability of Colo-679 or MelHO melanoma cells after 48h incubation with vincristine (10ng/ml), PLX4032 (10μM), or vincristine (10ng/ml) + PLX4032 (10μM) relative to non-treated control. * $P < 0.05$ relative to non-treated controls; # $P < 0.05$ relative to vincristine (10ng/ml) treatment; † $P < 0.05$ relative to PLX4032 (10μM) treatment.

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Suppl. Table 1. Influence of PLX4032, PLX4720 or the ABCB1 inhibitor PGP4008 on the concentration of the ABCB1 substrate vincristine that decreases the viability of ABCB1-expressing UKF-NB-3^{ABCB1} cells by 50% (IC₅₀).

	cell viability in the absence of vincristine (%)	IC ₅₀ vincristine (ng/mL) ¹	fold sensitisation relative to vincristine alone
PLX4032 (μM)			
0	100.00 ± 0.00	6.71 ± 0.67	1.00
0.625	91.56 ± 10.38	6.33 ± 1.72	1.06
1.25	82.88 ± 7.46	3.50 ± 0.39*	1.92
2.5	80.77 ± 4.14	1.28 ± 0.42*	5.24
5	89.86 ± 5.65	1.06 ± 0.20*	6.33
10	82.75 ± 14.95	0.89 ± 0.16*	7.54
20	90.69 ± 4.42	0.32 ± 0.10*	20.97
PGP4008 2.5μM	79.42 ± 14.29	0.16 ± 0.05*	41.94
PLX4720 (μM)			
0	100.00 ± 0.00	6.18 ± 0.17	1.00
0.625	100.61 ± 6.97	6.54 ± 1.98	0.94
1.25	89.23 ± 9.90	5.57 ± 0.35	1.11
2.5	81.50 ± 9.19	4.88 ± 1.36	1.26
5	100.00 ± 16.87	3.02 ± 0.22*	2.05
10	75.86 ± 7.00	2.47 ± 0.61*	2.50
20	76.00 ± 11.31	0.70 ± 0.05*	8.83
PGP4008 2.5μM	82.24 ± 20.45	0.12 ± 0.03*	51.50

¹ in the presence of the indicated PLX4032, PLX4720, or PGP4008 concentrations

* p < 0.05 relative to vincristine alone

Suppl. Table 2. Influence of PLX4032 or the ABCB1 inhibitor PGP4008 on the concentration of the ABCB1 substrate vincristine that decreases the viability of UKF-NB-3 cells by 50% (IC₅₀).

PLX4032 (μM)	cell viability in the absence of vincristine (%)	IC ₅₀ vincristine (ng/mL) ¹	fold sensitisation relative to vincristine alone
0	100.00 ± 0.00	0.14 ± 0.03	1.00
0.625	95.25 ± 7.88	0.14 ± 0.06	1.00
1.25	87.19 ± 9.71	0.17 ± 0.04	0.82
2.5	74.61 ± 8.69	0.12 ± 0.03	1.17
5	75.28 ± 7.79	0.13 ± 0.05	1.08
10	70.37 ± 6.21	0.15 ± 0.05	0.93
20	45.32 ± 8.17	0.13 ± 0.05	1.08
PGP4008 (μM)			
2.5	77.82 ± 14.09	0.12 ± 0.05	1.17

¹ in the presence of the indicated PLX4032 or PGP4008 concentrations**Suppl. Table 3.** Influence of PLX4720 or the ABCB1 inhibitor PGP4008 on the concentration of the ABCB1 substrate vincristine that decreases the viability of UKF-NB-3 cells by 50% (IC₅₀).

PLX4720 (μM)	cell viability in the absence of vincristine (%)	IC ₅₀ vincristine (ng/mL) ¹	fold sensitisation relative to vincristine alone
0	100.00 ± 0.00	0.17 ± 0.06	1.00
0.625	95.19 ± 12.38	0.16 ± 0.03	1.06
1.25	82.56 ± 9.96	0.16 ± 0.05	1.06
2.5	88.15 ± 11.16	0.19 ± 0.02	0.89
5	80.06 ± 9.22	0.20 ± 0.04	0.85
10	61.51 ± 12.87	0.15 ± 0.05	1.13
20	40.83 ± 10.33	0.18 ± 0.06	0.94
PGP4008 (μM)			
2.5	74.64 ± 16.51	0.15 ± 0.03	1.13

¹ in the presence of the indicated PLX4720 or PGP4008 concentrations**Suppl. Table 4.** Influence of PLX4032 or the ABCB1 inhibitor PGP4008 on the concentration of the ABCB1 substrate vincristine that decreases the viability of UKF-NB-3 cells transduced with an empty control vector (as comparison to UKF-NB-3^{ABCB1}) by 50% (IC₅₀).

PLX4032 (μM)	cell viability in the absence of vincristine (%)	IC ₅₀ vincristine (ng/mL) ¹	fold sensitisation relative to vincristine alone
0	100.00 ± 0.00	0.18 ± 0.06	1.00
0.625	92.86 ± 11.74	0.16 ± 0.04	1.13
1.25	80.49 ± 10.82	0.13 ± 0.07	1.38
2.5	81.19 ± 9.96	0.18 ± 0.01	1.00
5	78.67 ± 4.51	0.12 ± 0.08	1.50
10	72.57 ± 4.19	0.19 ± 0.02	0.95
20	40.67 ± 7.73	0.12 ± 0.04	1.50
PGP4008 (μM)			
2.5	76.68 ± 15.01	0.16 ± 0.06	1.13

¹ in the presence of the indicated PLX4032 or PGP4008 concentrations

Suppl. Table 5. Influence of PLX4720 or the ABCB1 inhibitor PGP4008 on the concentration of the ABCB1 substrate vincristine that decreases the viability of UKF-NB-3 cells transduced with an empty control vector (as comparison to UKF-NB-3^{ABCB1}) by 50% (IC₅₀).

PLX4720 (μM)	cell viability in the absence of vincristine (%)	IC ₅₀ vincristine (ng/mL) ¹	fold sensitisation relative to vincristine alone
0	100.00 ± 0.00	0.22 ± 0.07	1.00
0.625	90.26 ± 7.84	0.20 ± 0.08	1.10
1.25	96.88 ± 5.95	0.19 ± 0.03	1.16
2.5	77.89 ± 6.09	0.16 ± 0.06	1.38
5	75.31 ± 9.01	0.25 ± 0.04	0.88
10	66.28 ± 14.72	0.18 ± 0.05	1.22
20	35.16 ± 12.63	0.21 ± 0.05	1.05
PGP4008 (μM)			
2.5	79.65 ± 16.96	0.19 ± 0.06	1.16

¹ in the presence of the indicated PLX4720 or PGP4008 concentrations**Suppl Table 6.** Viability of the ABCB1-expressing cell lines UKF-NB-3^{ABCB1} or UKF-NB-3^{VCR}¹⁰ after treatment with vincristine, paclitaxel, actinomycin D (cytotoxic ABCB1 substrates), or cisplatin (no ABCB1 substrate) in the absence or presence of PLX4032 (10μM) or PLX4720 (10μM), determined by MTT assay after a 5 day incubation period.

Cell line	Concentration that inhibits cell viability by 50% (IC ₅₀)			
	vincristine (ng/mL)	paclitaxel (ng/mL)	actinomycin D (ng/mL)	cisplatin (ng/mL)
UKF-NB-3 ^{ABCB1}	6.05 ± 0.48	16.89 ± 1.65	0.70 ± 0.04	136 ± 16
+ PLX4032 (91 ± 3%) ¹	0.16 ± 0.05 ² (37.81) ³	2.22 ± 0.62 ² (7.61)	0.043 ± 0.004 ² (16.28)	111 ± 17 (1.23)
+ PLX4720 (76 ± 5%)	0.88 ± 0.23 ² (6.88)	6.18 ± 0.89 ² (2.73)	0.20 ± 0.03 ² (3.50)	132 ± 24 ² (1.03)
UKF-NB-3 ^{VCR} ¹⁰	34.42 ± 5.80	19.64 ± 2.07	3.09 ± 1.21	215 ± 26
+ PLX4032 (90 ± 3%)	1.23 ± 0.26 ² (27.98)	4.79 ± 0.63 ² (4.10)	0.51 ± 0.20 ² (6.06)	201 ± 10 (1.07)
+ PLX4720 (83 ± 10%)	4.79 ± 0.63 ² (7.19)	11.07 ± 1.53 ² (1.77)	0.84 ± 0.15 ² (3.68)	199 ± 23 ² (1.08)

¹ Cell viability in the presence of PLX4032 (10μM) or PLX4720 (10μM) alone relative to non-treated cells² p < 0.05 relative to the IC₅₀ value in the absence of PLX4032 and PLX4720³ Fold sensitisation relative to cytotoxic drug alone

Suppl. Table 7. Viability of UKF-NB-3 or UKF-NB-3^{control} (UKF-NB-3 cells transduced with an empty vector serving as control to UKF-NB-3^{ABCB1}) cells after treatment with vincristine, paclitaxel, actinomycin D (cytotoxic ABCB1 substrates), or cisplatin (no ABCB1 substrate) in the absence or presence of PLX4032 (10 μ M) or PLX4720 (10 μ M), determined by MTT assay after a 5 day incubation period.

Cell line	Concentration that inhibits cell viability by 50% (IC ₅₀)			
	vincristine (ng/mL)	paclitaxel (ng/mL)	actinomycin D (ng/mL)	cisplatin (ng/mL)
UKF-NB-3	0.14 \pm 0.05	1.68 \pm 0.12	0.083 \pm 0.002	115 \pm 3
+ PLX4032 (72 \pm 7%) ¹	0.12 \pm 0.03 (1.17) ²	1.51 \pm 0.30 (1.11)	0.080 \pm 0.007 (1.04)	103 \pm 13 (1.12)
+ PLX4720 (64 \pm 7%)	0.11 \pm 0.04 (1.27)	1.65 \pm 0.14 (1.02)	0.084 \pm 0.009 (1.02)	136 \pm 20 (0.85)
UKF-NB-3 ^{control}	0.12 \pm 0.02	1.38 \pm 0.17	0.12 \pm 0.03	82 \pm 3
+ PLX4032 (88 \pm 3%)	0.10 \pm 0.03 (1.20)	1.50 \pm 0.29 (0.92)	0.11 \pm 0.02 (1.09)	85 \pm 11 (0.96)
+ PLX4720 (75 \pm 4%)	0.13 \pm 0.02 (0.92)	1.56 \pm 0.23 (0.88)	0.13 \pm 0.03 (0.92)	99 \pm 21 (0.83)

¹ Cell viability in the presence of PLX4032 (10 μ M) or PLX4720 (10 μ M) alone relative to non-treated cells
² Fold sensitisation relative to cytotoxic drug alone

Table 3. ABCB1 expression in melanoma cells and the influence of the ABCB1 inhibitor PGP4008 (2.5 μ M) on the concentration of the ABCB1 substrate vincristine that decreases the viability of melanoma cells by 50% (IC₅₀).

Cell line	BRAF status	ABCB1 expression		IC ₅₀ vincristine (ng/ml)		fold sensitisation ²
			RFU ¹		+ PGP4008	
IPC298	wild-type	yes	27 \pm 9	2.74 \pm 0.39	0.75 \pm 0.42 ³ (79 \pm 3) ⁴	3.65
SK-Mel-30	wild-type	yes	88 \pm 15	4.57 \pm 1.49	0.92 \pm 0.33 ³ (91 \pm 10)	4.99

¹ RFU, relative fluorescence units (background fluorescence determined by the use of an isotype control was subtracted)² IC₅₀ vincristine/ IC₅₀ vincristine in the presence of PGP4008³ P < 0.05 relative to vincristine alone⁴ cell viability (% non-treated control) in the presence of PGP4008 alone